SCREENING PROCESS FOR ANTIBACTERIAL AGENTS

5   The present invention relates to the isolation of
     subpopulations of stationary phase bacteria which
     exhibit resistance to conventional antibacterial agents,
     to the use of such resistant subpopulations in screening
     processes for the identification of new and improved
10   antibacterial agents, to novel antibacterial agents
     identified thereby, and to therapeutic applications of
     such antibacterials.

        Tuberculosis remains a serious disease throughout
     the world and it has been estimated that deaths
15   resulting from tuberculosis account for 7% of the total
     number of deaths from infectious diseases. As discussed
     in US Patent No. 5,700,925, the vast majority of
     individuals who become infected with *Mycobacterium
     tuberculosis* do not develop symptomatic tuberculosis but
20   do exhibit a positive reaction to the tuberculin skin
     test. In such infected hosts, some bacteria persist in
     a dormant or latent state in which they are
     substantially resistant to antimicrobial drugs. Over a
     lifetime, up to 10% of these infected but asymptomatic
25   individuals may go on to develop tuberculosis, often
     many years after primary infection, when the dormant
     bacilli become activated and start to grow. This can
     result in pulmonary tuberculosis and other variant forms
     of the disease. Factors which predispose towards
30   activation of the dormant organism and manifestation of
     the diseased state include poverty, poor living
     conditions, malnutrition, immune deficiency or immune
     suppression.

        In general in the treatment of tuberculosis,
35   antimicrobial therapy using antibacterial agents such as
     rifampicin, isoniazid and/or pyrazinamide is relatively
     successful against actively growing bacteria. Such

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therapy is ineffective, however, against bacteria which
remain dormant or which, having undergone a growing
phase, re-enter a dormant phase. This causes particular
problems in the clinical treatment of tuberculosis
5   sufferers and carriers because the resistance of the
dormant organisms necessitates long term
chemotherapeutic care. Such long term treatment,
typically of six months duration, is unsatisfactory
since it is expensive, may result in poor patient
10   compliance and may encourage the development and
emergence of antibiotic resistant strains of bacteria
over and above the *M. tuberculosis* targetted during
therapy.
       It is believed that most pathogenic bacteria for
15   example *Staphylococcus aureus, Haemophilus influenzae,
Streptococcus pyogenes, Streptococcus gordonii* and *E.
coli*, possess a similar substantially antibacterial
agent-resistant subpopulation which may act as a pool
for reinfection during or after chemotherapy. These
20   persistent bacteria are usually drug-sensitive at
relapse, indicating that their resistance to
chemotherapy is phenotypic rather than genetic.
       In order to investigate whether the metabolism of
such persistent bacteria is switched off with no cell
25   division (i.e. is spore-like) or is active (i.e. so that
the cells will contain markers of metabolism such as
mRNA), we have studied *M. tuberculosis* in an *in vitro*
stationary phase model obtained by long term culturing
of the organisms in a microaerophilic gradient in which
30   the stationary phase organisms are viable. Such
stationary phase bacteria were found to be resistant to
rifampicin at the normal minimum inhibitory
concentration (MIC) level of 0.1 μg/ml (as assessed with
bacteria at concentration of 10^6-10^7 cfu/ml). Viability
35   of *M. tuberculosis in vivo* before and after treatment
with anti-tuberculosis agents has also been examined.
Viable persistent non-culturable bacteria were present

after treatment with antibacterials, whose dormancy was reversed when the host animals were subjected to steroid therapy.

The present invention is based on the unexpected and surprising finding that, whilst treatment of such stationary phase bacteria with antibacterial agents such as rifampicin at concentrations greatly exceeding the minimum inhibitory concentration reduces plate counts to zero colony forming units (CFU's), there remains a small number of persistent organisms which are detectable by, for example, broth dilution counting. These resistant subpopulations are phenotypically resistant to rifampicin, since they become sensitive to rifampicin at normal MIC levels upon resumption of growth.

Analogous studies using stationary phase E. coli and S. aureus model systems and treatment thereof with kanamycin and ampicillin respectively have yielded similar results.

In a first aspect, the present invention thus provides a method of preparing a phenotypically antibiotic-resistant subpopulation of stationary phase bacteria comprising at least the steps of:

(i) growing a bacterial culture to stationary phase; and

(ii) treating said stationary phase culture with one or more antibacterial agents at a concentration and for a time sufficient to kill growing bacteria, thereby selecting a phenotypically antibiotic-resistant subpopulation.

As referred to herein, the "phenotypically antibiotic-resistant" bacteria refers to a portion of the stationary phase bacteria which remain metabolically active (e.g. as examined by transcriptional activity) after treatment of the stationary phase bacteria with a particular antibiotic (or more than one antibiotic) administered at a high dosage, namely at least 10 times higher than MIC. This subpopulation is however

susceptible to treatment with the antibiotic when not in
the stationary phase. It will be appreciated that a
subpopulation of bacteria resistant to a particular
antibiotic may be distinct to a different antibiotic resistant
5   to a different antibiotic.

As referred to herein "kill" refers to a loss of
viability as assessed by a lack of metabolic activity.
In order to achieve this effect, antibiotic is
administered to the stationary phase for an appropriate
10   time and at an appropriate concentration. Conveniently
antibiotic is applied at a concentration of from 10 to
10^4 x MIC, e.g. at a concentration of 25 to 150μg/ml
(e.g. 50 to 100μg/ml) with the bacteria at a
concentration of 10^5 to 10^9 bacteria/ml (e.g. 10^6 to 10^7
15   bacterial/ml). Conveniently bacteria are treated with
the anti-bacterial agent(s) for 1-10 days, e.g. 3-5
days.

The nature of the antibacterial agent employed may
depend on the particular bacteria being investigated.
20   The use of antibacterial agents such as rifampicin which
target RNA polymerase, for example at concentrations of
10^2, 10^3 or 10^4 times the normal MIC level, has been
found convenient. In a representative embodiment of
this aspect of the invention, treatment of stationary
25   phase *M. tuberculosis* at a level of 10^6 bacteria/ml for
one day with 100 μg/ml of rifampicin (MIC x 10^3) resulted
in a phenotypically resistant subpopulation of 10^2
bacteria/ml. Alternatively, *M. tuberculosis* may be
treated with pyrazinamide. In further embodiments,
30   treatment of stationary phase *E. coli* or *S. aureus* ($\tilde{}$ 10^9
bacteria/ml) with 50 μg/ml kanamycin or 100 μg/ml
ampicillin for 3 days results in a phenotypically
resistant subpopulation of $\tilde{}$ 10^5 bacteria/ml.

The phenotypically antibiotic-resistant
35   subpopulation may be seen as representative of the
persistent bacteria which remain metabolically active *in
vivo* and which can result in relapse or onset of

disease.   Of interest therefore in particular are
antibiotic-resistant populations from bacteria which
exhibit dormancy *in vivo*, e.g. bacteria from the genera
Staphylococcus, Escherichia, Hemophilus and

5   *Mycobacterium*.   Particularly preferred are
subpopulations from the bacteria *Staphylococcus aureus,
Escherichia coli, Haemophilus influenzae, Streptococcus
pyogenes, Streptococcus gordonii* and *Mycobacterium
tuberculosis.*

10   In particularly preferred features, the bacteria
are *Mycobacterium tuberculosis, Escherichia coli* and
Staphylococcus aureus treated with rifampicin, kanamycin
and ampicillin, respectively.
       Such phenotypically resistant subpopulations of

15   stationary phase bacteria, obtainable by treating
stationary phase bacteria with a high dosage of an
antibacterial agent, constitute a further feature of the
present invention.
       On the basis that the phenotypically resistant

20   subpopulations of the invention mimic the behaviour of
dormant bacteria *in vivo*, they constitute valuable tools
in screening procedures designed to identify potentially
valuable new antibacterial agents.   It will be
appreciated that antibacterial agents having activity

25   against such phenotypically resistant subpopulations may
have great therapeutic potential in the treatment of
diseases such as tuberculosis in which dormant bacteria
provide a pool for reinfection.
       Thus, in a further aspect, the present invention

30   provides a process for assessing the antibacterial
activity of a test compound or agent or for identifying
or isolating a compound or agent having antibacterial
activity against stationary phase bacteria comprising
the steps of:

35   (i) growing a bacterial culture to stationary
phase;
           (ii) treating said stationary phase culture with

one or more antibacterial agents at a concentration and
for a time sufficient to kill growing bacteria, thereby
selecting a phenotypically resistant subpopulation;

    (iii) incubating a sample of said phenotypically
5   resistant subpopulation with one or more test compounds
or agents; and

    (iv) assessing any antibacterial effects against
said phenotypically resistant subpopulation and
optionally isolating a compound or agent exhibiting
10 antibacterial activity.

    Such a process facilitates the rapid screening of
large numbers of compounds quickly and efficiently. The
compounds to be screened may be any chemical compounds
and may be already known or may themselves be novel.
15 Especially suitable candidate compounds are the products
of combinatorial chemistry, phage display libraries,
natural products, synthetic, semi-synthetic or natural
homologues, analogues, derivatives and precursors of
known antimicrobial agents or other pharmaceuticals.
20 Such homologues, analogues, derivatives and precursors
are entities which are related to or derived from
naturally occurring antimicrobials or other
pharmaceuticals, e.g. where this is a protein or peptide
by single or multiple amino acid substitution, addition
25 and/or deletion or alteration of the level of
glycosylation, or if the antimicrobial is a chemical
compound by chemical derivatization, e.g.
esterification, addition of various substituents,
methylation etc. Preferably the above described
30 modifications will not affect the principal function of
the molecule, e.g. they will retain antimicrobial
activity although the extent of this activity may be
modified.

    To perform the test, conveniently 1-1000μl, e.g.
35 50-300μl of the prepared antibiotic-resistant bacteria
(which is washed prior to use) containing 10^3 to 10^6
bacteria/ml are added to the sample to be tested e.g 0.1

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to 100µg, preferably 1-20µg. These are incubated together at a temperature suitable for metabolic activity, preferably 37°C. Antibacterial effects are then assessed at one or more time points varying from 1 day to 4 weeks, preferably once weekly for 4 weeks.

The term "assessing" as used herein in reference to antibacterial effects includes both quantitation in the sense of obtaining an absolute value of the extent of anti-bacterial activity, and also obtaining a semi-quantitative assessment or other indication, e.g. an index or ratio, of the amount of anti-bacterial activity in the sample. Assessing includes the identification of both positive effects on bacterial activity. The identification and optional isolation of agents or compounds which exhibit anti-bacterial effects is however preferred. Such anti-bacterial effects may be bacteriocidal or bacteriostatic in nature, although the former is preferred.

The existence of anti-bacterial activity may be ascertained by comparing the level of metabolically active or live bacteria in the test sample to control samples. This assessment may be achieved by any appropriate technique which identifies metabolic activity. Thus for example, transcriptional or translational activity may be assessed in respect of one or more genes, e.g. by monitoring gene transcript levels, or more generally, e.g. by monitoring the incorporation of particular bases or amino acids. Alternatively other techniques may be used, e.g. the ability to form colonies may be assessed, e.g. cfu counts may be determined after resuscitation in liquid culture even though direct plating on solid media without intermediate liquid medium culture may fail to yield cfu. Compounds of interest may optionally be re-screened at different concentrations, for example to establish the MIC level.

Compounds or agents having anti-bacterial activity

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are considered to be those which, at the concentration
used, adversely affect the metabolic activity of the
phenotypically antibiotic-resistant subpopulation,
preferably by reducing metabolic activity to less than
5   50%, especially preferably less than 25%, e.g. less than
10% compared to untreated control samples.

Candidate compounds identified in such a manner,
for example exhibiting a minimum inhibitory
concentration of 10 μg/ml or less, preferably 1 μg/ml or
10 less, especially preferably 0.1μg/ml (as assessed with
bacteria at a concentration of 10^6 to 10^7 cfu/ml) in
respect of the resistant subpopulation, may then be
subjected to further analysis, efficacy and toxicity
tests etc.

15   The invention furthermore extends to kits for
performing the screening method of the invention. Thus
the present invention provides a kit for assessing the
antibacterial activity of a test compound or identifying
an antibacterial agent, comprising at least the
20 following:
i)   a phenotypically antibiotic-resistant
subpopulation of stationary phase bacteria as defined
herein.

Preferably the kit also contains appropriate
25 buffers and solutions (particularly for maintaining the
bacteria, e.g. including appropriate antibacterial
agents) for assessing antibacterial effects of the test
compound or agent by ascertaining metabolic activity of
the bacteria. Additionally, the kit may also include
30 means for standardization of the assay or for
comparative purposes.

Compounds or antibacterial agents identified or
isolated by the above mentioned process are preferably
produced on a larger scale for further testing or use.
35 Depending on the nature of the test compounds this may
be achieved using synthetic or natural processes or a
combination of both. For example, where the test

compound is a peptide this may be produced synthetically
by peptide synthesis or may be produced by expression of
an appropriate DNA molecule. Thus in a preferred
aspect, the present invention provides a process for
5   preparing an agent having anti-bacterial activity
against stationary phase bacteria wherein said agent
identified according to the process described herein is
amplified.

In a further aspect, the present invention is
10   directed towards novel chemical compounds which exhibit
bacteriostatic or bactericidal effects on an
antibacterial agent resistant subpopulation of
stationary phase bacteria, e.g. a rifampicin-resistant
subpopulation of *M. tuberculosis*, a kanamycin-resistant
15   subpopulation of *E. coli* or an ampicillin-resistant
subpopulation of *S. aureus*. In particular, the present
invention provides antibacterial agents identified by
the above described process.

It is believed that resistance of persistent
20   bacteria to antibacterial agents may be due to a
decrease in cell wall permeability and/or the use of
alternative sigma factors (such as *sigB*). New
antibacterial agents which have the effect of increasing
cell wall permeability or inhibiting the use of
25   alternative sigma factors are therefore especially
preferred.

Anti-bacterial agents identified according to the
invention have particular utility in the treatment of
diseases or disorders in which dormant bacteria provide
30   a pool from which symptomatic disease may be initiated
or restarted. In particular diseases resulting from
pathogenic bacteria of the genera *Staphylococcus*,
Escherichia, *Hemophilus* and *Mycobacterium* may be
targetted. Anti-bacterial agents having activity
35   against the persistent bacteria may be used during
symptomatic or asymptomatic stages of the disease or
condition, when those persistent bacteria are dormant or

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in a non-dormant state, and optionally may be used in
conjunction with agents directed to non-dormant
bacteria. "Non-dormant bacteria" as used herein
includes bacteria which at the time are in a non-dormant
state, ie. are actively growing, but may or may not at
other times exhibit a dormant state.

In another aspect therefore, the present invention
is directed towards a composition or formulation
comprising a novel antimicrobial e.g. antibacterial
agent or chemical compound as described herein and a
pharmaceutically acceptable diluent or excipient.

By "pharmaceutically acceptable" is meant that the
ingredient must be compatible with other ingredients of
the compositions as well as physiologically acceptable
to the recipient.

Pharmaceutical compositions according to the
invention may be formulated in conventional manner using
readily available ingredients. Thus, the active
ingredient may be incorporated, optionally together with
other active substances, with one or more conventional
carriers, diluents and/or excipients, to produce
conventional galenic preparations such as tablets,
pills, powders, lozenges, sachets, cachets, elixirs,
suspensions, emulsions, solutions, syrups, aerosols (as
a solid or in a liquid medium), ointments, soft and hard
gelatin capsules, suppositories, sterile injectable
solutions, sterile packaged powders, and the like.

Example of suitable carriers, excipients, and
diluents are lactose, dextrose, sucrose, sorbitol,
manitol, starches, gum acacia, calcium phosphate,
aglinates, tragacanth, gelatin, calcium silicate,
microcrystalline cellulose, polyvinylpyrrolidone,
cellulose, water syrup, water, water/ethanol,
water/glycol, water/polyethylene glycol, propylene
glycol, methyl cellulose, methylhydroxybenzoates, propyl
hydroxybenzoates, talc, magnesium stearate, mineral oil
or fatty substances such as hard fat or suitable mixture

thereof.

In the clinical management of tuberculosis and other disease states where the establishment and existence of dormant bacteria is problematic, it may be advantageous to administer a combination of antibacterial agents which are respectively directed towards the different growth phases of these organisms. Thus, for example, an appropriate chemotherapeutic approach to the treatment of tuberculosis and similar diseases would be to administer to a patient one or more antibacterial agents directed against the growing or log-phase of the microorganisms and one or more antibacterial agents directed against the dormant or stationary phase population.

Thus, a preferred formulation according to the present invention comprises at least one antibacterial agent which has activity against actively growing bacteria and at least one antibacterial agent or chemical compound (as described herein) having activity against the phenotypically resistant subpopulation of the stationary phase of said bacteria. Such formulations may be presented as a combined preparation for simultaneous, separate or sequential use in the treatment of bacterial infections such as tuberculosis.

Such formulations may be administered during symptomatic or asymptomatic stages of the disease, ie. the antibacterial agent against the resistant sub-population need not necessarily be used when these bacteria are in the dormant phase but may be used when they are actively growing.

As used herein antibacterial agents which have activity against actively growing bacteria exhibit antibacterial effects as described hereinbefore against bacteria during the log-phase.

In a further aspect, the present invention provides the compounds or agents, identified as effective against phenotypically resistant subpopulations of stationary

phase bacteria, e.g. by the above described screening
process, or compositions containing them for use in the
treatment of bacterial infection. Conveniently,
bacterial infections which may appropriately be treated
5   are those in which persistent bacteria would remain
after infection even when the infected macroorganism is
asymptomatic, e.g. before the onset of disease or after
treatment with known antibacterials. These bacteria may
be in the dormant phase and thus in a preferred aspect
10   the bacterial infection to be treated involves (or could
potentially involve) dormant bacteria, insofar as the
infection is typified or characterized by a
subpopulation of persistent bacteria which remain in
asymptomatic patients in the absence of actively growing
15   bacteria, ie. which may enter a dormant phase after
infection.

    In yet another aspect, the present invention
relates to the use of compounds or agents identified as
effective against phenotypically resistant
20   subpopulations of stationary phase bacteria, e.g. by the
above screening process, or compositions containing them
in the preparation of a medicament for the treatment of
bacterial infections.

    The use of such antibacterial compounds identified
25   by the above process in the treatment of bacterial
infections (preferably those involving dormant bacteria)
constitutes another aspect the present invention.

    Alternatively viewed, the present invention
provides a method of treatment of a bacterial infection
30   comprising administering to a patient in need of such
therapy an effective amount of an antibacterial agent or
compound directed towards the stationary phase of
growth, optionally in the presence of one or more
antibacterial agent directed towards the growing phase
35   of said organism.

    In therapeutic methods according to the invention,
preferably, the animal to be treated is a mammal,

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especially preferably a human.

As mentioned above, administration may be made when
the diseased individual is symptomatic or asymptomatic.
Although the antibacterial agents of the invention may
5   be identified as effective against dormant, persistent,
bacteria, ie. in the stationary phase of growth, the
agents of the invention may be administered when these
bacteria are actively growing.

Administration of the composition may take place by
10   any of the conventional routes, e.g. orally, rectally or
parenterally such as by intramuscular, subcutaneous,
intraperitoneal or intravenous injection.

The active ingredient in such compositions may
comprise from about 0.01% to about 99% weight of the
15   formulation, preferably from about 0.1 to about 50%, for
example 10%.   The precise dosage of the active
ingredient to be administered and the length of the
course of treatment will, of course, depend on a number
of factors including for example, the age and weight of
20   the patient, the specific condition requiring treatment
and its severity, and the route of administration.

In each aspect of the invention, the stationary
phase bacteria which are the target of the antibacterial
agents may include pathogenic bacteria such as
25   *Staphylococcus aureus, Haemophilus influenzae,*
Streptococcus pyogenes, Streptococcus gordonii,
Escherichia coli and particularly preferably,
Mycobacterium tuberculosis.

30   The following non-limitative Examples serve to
illustrate the invention.   In the accompanying drawings:
Fig. 1 shows growth curves for *E. coli* and *S.*
aureus over a period of 10 days;
Figure 2 shows the viability of *E. coli* after
35   treatment with 50 µg/ml kanamycin during stationary
phase and log phase growth;
Figure 3 shows the viability of *S. aureus* after

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treatment with 100 μg/ml ampicillin during stationary
phase and log phase growth.

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EXAMPLE 1

Growth of M. tuberculosis and selection for a
phenotypically but not genetically rifampicin resistant
5   population of cells

M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth
containing 0.05% Tween® 80 supplemented with 10% ADC,
without agitation or other disturbance for up to 100
10   days, in accordance with the procedure of Wayne (1976)
Amer. Rev. Resp. Dis. 114: 807-811. Where the
stationary phase organisms were viable they were
resistant to MIC levels of 0.1 μg/ml rifampicin.

15   Sixty 10 ml samples of the cultures were vortexed with
glass beads for 3-5 minutes, followed by sonication in a
water bath sonicator for less than 5 minutes. All the
cultures were pooled into a 500 ml sterile bottle with
screw cap.
20

Prior to addition of rifampicin, samples of the culture
were taken to check purity, viability and metabolic
activity as follows:

25   1.   2 x 100 μl for CFU counts
       2.   2 x 5 ml for broth counts
       3.   2 x 10 ml for [^{3}H]uridine counts
       4.   2 x 5 ml for [^{35}S]methionine counts
       5.   10 ml for RNA extraction for RT-PCR
30   6.   10 ml for drug-free control
       7.   20 μl for blood agar to check sterility

Selection and detection of a rifampicin-resistant
subpopulation
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50 ml of 1000 μg/ml rifampicin was added to 500 ml of
the above culture to obtain a final rifampicin

concentration of 100 μg/ml, and the culture was
incubated at 37°C for 5 days. The cells were then
harvested in sterile 50 ml tubes by centrifugation at
5000g for 15 minutes, then washed twice in sterile PBS
5   containing 0.05% Tween® 80 and Selectitab® antibiotics
(which comprises a combination of antibacterial agents
which kills most bacteria but not *M. tuberculosis*, thus
preventing *M. tuberculosis* from becoming overgrown by
faster growing bacteria). The cells were resuspended in
10   500 ml fresh 7H9 medium.

Sterility of the culture was checked before further
experiments. 2 x 100 μl samples of the culture was
added to blood agar plates in duplicate which were
15   incubated at 37°C overnight. The cell suspension was
kept at 4°C overnight. Contaminated cultures should
always be discarded.

It was found that this treatment with high levels of
20   rifampicin reduced plate counts to zero colony forming
units but resulted in a small number of persisting
organisms which were detectable by broth dilution
counting. Table 1 shows a typical example of such
selection where 10^2 bacteria/ml remained from 10^6 after
25   one day of rifampicin treatment. From this, it appears
that *M. tuberculosis* has at least two populations of
stationary phase organisms: the first is killed by high
dose rifampicin and the second 'persists' and is
phenotypically resistant. Upon resumption of growth,
30   the rifampicin-resistant stationary phase subpopulation
becomes sensitive to rifampicin at the normal MIC level
of 0.1 μg/ml.

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EXAMPLE 2

Characterisation of the rifampicin-resistant
subpopulation *in vitro*

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Methods

M. *tuberculosis* was grown in 7H9 medium containing 0.05%
of Tween® 80 supplemented with 10% ADS (Difco
Laboratories) without shaking for 100 days. Rifampicin
10   was added to the cultures at a final concentration of
100 μg/ml for 5 days. Viability was estimated at one
day intervals. The cells were thoroughly washed and 100
μl of samples from 10-fold dilutions of the cultures
were added to triplicate plates of Middlebrook 7H11
15   medium supplemented with OADC (Difco Laboratories).
Colony forming units (CFU's) were counted after
incubation of the plate for 3 weeks at 37°C. Broth
counts were performed at 10-fold dilutions by adding 1
ml of the sample to 9 ml of 7H9 medium. Viability was
20   estimated by examining growth in the diluted cultures
after incubation for 6 weeks at 37°C.

For incorporation of radioactive uridine, 10 ml of
the culture for each time point was washed twice to
remove the remaining rifampicin and then resuspended in
25   10 ml 7H9 medium followed by incubation with 10 μCi/ml
of [^{3}H]-uridine for 20 hours. For 5* the culture was not
washed after incubation with rifampicin and 10 μCi/ml of
[^{3}H]-uridine was added to the culture which was further
incubated in the presence of rifampicin for 20 hours.
30   The RNA was extracted by methods known in the art. The
RNA [^{3}H]-uridine incorporation was determined as counts
per minute of trichloroacetic acid precipitated RNA.
The results were confirmed in three independent
experiments.

35

The metabolic state of bacilli before and after
rifampicin treatment was also assessed by examining

total RNA which was extracted. RT-PCR was performed on
the following genes: rpoB which encodes the rifampicin
target (RNA polymerase sub-unit B); 16 kDa protein (α-
crystallin homolog); 16S rRNA and two *Sigma 70*
5   homologues, sigA and sigB.

Results
The results are shown in Table 1.

10 Table 1: Incorporation of [³H]-uridine into *M.*
    tuberculosis after addition of rifampicin

Days in rifampicin	Plate counts	Broth counts	[³H]-uridine
0	6.6×10^5	10^6	74682±630
1	0	10^2	2228±88
2	0	10^2	2316±120
3	0	10^2	2430±54
4	0	10^2	2318±126
5	0	10^2	2388±20
5*	0	10^2	518±10
0	0 (heat-killed)	0	180±19

25 Samples of the rifampicin resistant subpopulation of
    cells were analysed and the cell population further
    characterised. The population was shown to be
    metabolically active by virtue of transcriptional and
    translational activity detected in the population. All
30 the genes which were examined were found to be
    transcribed in the stationary phase. After 5 days of
    rifampicin treatment which reduced the cfu to zero, the
    16S rRNA was not diminished and transcripts for *rpoB*,
    16K and *SigB* could still be detected, albeit at reduced
35 levels, whilst the transcript for *SigA* was not seen.

    Broth dilution counts of rifampicin treated cultures

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(days 1-5) gave values of 10^2 bacteria/ml (Table 1)
indicating the presence of bacteria capable of growth.
However, in spite of the four log drop in broth dilution
counts after rifampicin treatment, the RT-PCR signal for

5   *16S*, *sigB*, *16K* and *rpoB* was several log fold above that
which would be expected for such low numbers of broth
positive surviving bacteria. In order to quantitate the
amount of 16S rRNA which was present, the rRNA level was
measured by Northern blotting before and after treatment

10 with rifampicin (data not shown). Densitometric
analysis revealed that about half of the signal
intensity remained after 7 days of treatment with the
antibiotic in both log-phase and stationary
organisms. This suggests that rRNA is very stable in

15 rifampicin-treated cultures. In contrast, the mRNAs
were much less stable than rRNA. The half-lives of
mRNAs for *16K* and *sigB* were measured in both log-phase
and long term stationary phase cultures and were shown
to be between 2-3 mins. Thus, it is unlikely that the

20 RT-PCR results are accounted for by long term stable
mRNA and suggest active transcription. This was tested
by first treating organisms with rifampicin, and then
incubating these bacteria simultaneously with rifampicin
and [³H]-uridine. In the presence of rifampicin the

25 bacteria incorporated [³H]-uridine indicative of active
transcription in persisting bacteria, even in the
presence of a high concentration of rifampicin and shows
that the bacteria are actively metabolising.

30 The cells were shown to be very responsive to changes in
their environment. When rifampicin was removed and
replaced by growth medium alone, the level of
transcription of three genes (*16K*, *sigA* and *rpoB*
transcripts) analysed increased by 5- to 10-fold after

35 12 hours. Radioactive uridine incorporation also
increased about 5-fold after removal of rifampicin and
incubation with medium alone (Table 1 - compare counts

- 20 -

at 5' and 5 days). This suggested that the level of
transcription increased rapidly under these
circumstances. The data do not exclude the possibility
that the organisms replicate in the presence of fresh
5   medium, although this would only account for a two-fold
rise in transcription in 12 hours, since the generation
time of *M. tuberculosis* is about 20 hours. The data
support a hypothesis that, in this *in vitro* model of
drug resistant stationary-phase bacteria, there is a
10   major population of bacteria that are actively
transcribing RNA and are environmentally reactive, yet
remain plate culture negative.

To distinguish between phenotypic and genotypic
15   resistance the bacteria were thoroughly washed after
rifampicin treatment and cultured in liquid 7H9 medium
for 6 weeks. In four separate experiments *M.
tuberculosis* was invariably grown. These bacteria were
sensitive to 0.1 µg/ml rifampicin and were negative for
20   rifampicin resistant mutations in rpoB as detected by
RT-PCR and hybridisation with oligonucleotide probes
(Immunogenetics N.V., Netherlands; data not shown).
This indicates that the resistance is phenotypic in this
model.
25

It is unlikely that the mechanism of induced resistance
depends on mutation or reduced levels of expression of
rpoB mRNA (i.e. resulting in a reduced level of drug
target) because transcription continues even in the
30   presence of rifampicin (Table 1).

Characterisation of the antibacterial-resistant
subpopulation *in vivo*
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Methods
The antibacterial-resistant subpopulation was examined

in vivo according to the Cornell dormancy model in which
murine chronic tuberculosis is induced by chemotherapy.
M. tuberculosis was grown in BALB/c mice weighing 18-20g
which were infected intravenously with 2×10^5 cfu of a
5   mouse-passaged, virulent H37Rv strain of *M.
tuberculosis*. Spleens and lungs were removed rapidly
after sacrifice and sterile autopsy at time point -2
week and 0 week (2 weeks after infection); portions of
the organs were immediately frozen in liquid N_2.

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Cfu counts of viable *M. tuberculosis* were done by
grinding the spleen and lungs in 5ml water in motor-
driven polytetrafluorethylene/glass grinders and setting
up counts from serial dilution of the homogenate on
15   plates of selective 7H11 medium (Difco, Detroit).

Treatment was then given for 14 weeks with 1000 mg
pyrazinamide/kg and 25 mg isoniazid/kg body weight in
the pelleted diet when the "sterile state" was reached.
20   A sample of mice was also sacrificed at the 7 week
timepoint. A further sample was then sacrificed at
timepoint 14, and the entire organ homogenates were
cultured in selective Kirchner liquid medium (Lab M,
Amersham). Culture media were made selective for *M.
25   tuberculosis* by the addition of 200 U polymyxin B/ml,
100 μg carbenicillin/ml, 20 μg trimethoprim/ml and 10 μg
amphotericin/ml. During a further 8 weeks the remaining
sample of mice were administered steroid therapy.

30   Results
RT-PCR of RNA from lungs removed before treatment
revealed transcripts for *rpoB*, *sigA*, *sigB*, *16K* and *16S*
rRNA. Significantly, bacterial mRNA for *sigB* and *16K*
was detected in lungs from day 91 (13 weeks) post
35   treatment and the level of *16S* rRNA was not reduced
compared to the pre-chemotherapy level. It is
surprising to find any mRNA at all after 13 weeks of

chemotherapy since mRNA is depleted within a few hours.
However, since we were not able to measure the half-life
of mRNA *in vivo* or the uptake of [³H]-uridine into RNA,
we could not discriminate between unstable and stable
5   mRNAs. Nevertheless, the data suggest that, substantial
numbers of persisting bacilli are present in the lungs
at day 91 which may be metabolically active because they
contain mRNA, but are not culturable. All lungs used
for RT-PCR were culture negative.

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As in the *in vitro* model, the *sigA* transcripts were not
detectable in mouse persisters and this may be due to
low abundance of this mRNA leading to levels below
detectable limits, or it could be due to insensitive
15 primers. Current data do not discriminate between these
possibilities. The *sigF* transcripts (data not shown)
were detected in the Cornell mice but the bands were
very weak. All controls were performed to ensure RT-PCR
products derived from mRNA and not from contaminating
20 genomic DNA.

Two weeks after infection, at time 0 week, a sample of 4
mice yielded 2-5 x 10⁷ cfu/lungs or spleen. Four further
mice were sacrificed at 7 and 14 weeks. Of these 8 mice
25 two yielded a positive organ culture, one from the
spleen only and one from the lungs. However, mice in
this "sterile" state contain large amounts of *M.
tuberculosis* DNA which is estimated to be equivalent to
10⁵ bacteria per organ. Upon treatment of the mice with
30 steroids, spleens and lungs yielded positive cultures
indicating reactivation from a dormant, non-culturable
and drug insensitive state.

Without wishing to be bound by theory it is believed
35 that the mechanism of resistance of the persistent
bacteria to antibacterial agents may be due to a
decrease in cell wall permeability (EDTA permeabilizes

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persister cell walls and increases the efficacy of
rifampicin, data not shown) and the use of alternative
sigma factors (such as *sigB*) which confer insensitivity
to the rifampicin-mediated inhibition of transcription.

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Susceptibility assessment of drug libraries

Sterile 0.7 ml labelled transparent plastic snap-capped
tubes containing 1-20 μg of the compound to be tested

10   are used. The compound is dissolved in 0.25 ml of
sterile distilled water or other appropriate diluent.
0.25 ml of the rifampicin treated culture is added to
each tube in the class I cabinet in a category III
safety containment laboratory with care being taken to

15   avoid contamination. The tubes are incubated at 37°C.
The drug effects are examined by CFU counts by addition
of 2 X 50 μl of each sample to 7H11 agar plates in
duplicate including 2 drug-free controls at 1 week
intervals. A series of 10-fold dilutions of the samples

20   may be required, which is made in 7H9 broth with 0.05%
Tween 80 but without ADC, then the diluted samples are
plated on 7H11 agars and the concentration effect of the
candidate compounds determined.

25   EXAMPLE 3

Selection of phenotypically but not genotypically
resistant subpopulations of *E. coli* and *S. aureus* using
kanamycin and ampicillin respectively

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Growth of *Escherichia coli* and *Staphylococcus aureus*

Escherichia coli K12 and *Staphylococcus aureus* are grown
in 10 ml of nutrient broth No. 2 (Oxoid) with continuous

35   shaking at 120 rpm for 10 days. Viability of the
bacteria is estimated by colony forming unit counts at 2
hours intervals for the first 24 hours and 12-24 hours

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afterward. From serial 10-fold dilutions of the
experimental cultures, 100 μl samples are added to
triplicate plates of nutrient agar plates (Oxoid).
Colony forming units (CFU) are counted after incubation
5   of the plates at 37°C for 24 hours.

Selection of persistent bacteria by antibiotics

Ampicillin and kanamycin are added to 5-day stationary-
10   phase cultures of E. coli and S. aureus respectively to
a final concentration of 100 μg/ml and 50 μg/ml
respectively for 3 days. After 3 days of antibiotic
treatment, the cells are washed with sterile distilled
water 3 times, then resuspended in 10 ml fresh nutrient
15   broth. Viability is estimated by CFU counts and broth
dilution counts. Broth dilution counts are performed in
a serial 10-fold dilution in nutrient broth, then 1 ml
of each dilution in added into 9 ml of nutrient broth in
30-ml universal tubes in triplicate. The growth curves
20   for E. coli and S. aureus over the 240 hour period in
the absence of antibiotics is shown in Fig. 1. The
effect of antibiotic exposure on the E. coli and S.
aureus during both log and stationary phase of growth is
shown in Figs. 2 and 3. Treatment of the cultures
25   during stationary phase results in the selection of an
antibiotic resistant subpopulation of cells.